Characterization of Binding Components for Progesterone and 5α-Pregnane-3,20-dione in the Hamster Uterus
(3α-hydroxy-5α-pregn-20-one/cytosol/hormone receptor/steroid metabolism/dihydroprogesterone)

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ABSTRACT In the hamster uterus, a specific progesterone (pregn-4-ene-3,20-dione) receptor has been identified in the cytosol fraction. In the present study, we examined hamster uterine cytosol for the possible existence of specific macromolecules that bind the progesterone metabolite, 5α-pregnane-3,20-dione. When cytosol was analyzed by density-gradient centrifugation with sucrose-glycerol gradients and by Scatchard plot analysis of [3H]5α-pregnane-3,20-dione binding data, there was no evidence of specific binding components for this metabolite. In vivo treatment of proestrous hamsters with unlabeled progesterone, 5α-pregnane-3,20-dione, or cortisol for 1 hr revealed that only progesterone caused the depletion of progesterone-receptor sites from the uterine cytosol fraction. Incubation of uterine strips which had been preloaded with two different concentrations of [3H]-progesterone demonstrated that progesterone was metabolized to 5α-pregnane-3,20-dione and to a greater extent to 3α-hydroxy-5α-pregn-20-one. The accumulation of 5α-pregnane-3,20-dione during progesterone metabolism appeared to be related to the availability of nonspecifically bound hormone. These studies (i) strongly suggest there is no specific receptor system for 5α-pregnane-3,20-dione in the uterine cytosol fraction, (ii) confirm the existence of a specific progesterone receptor in uterine cytosol, and (iii) provide evidence that progesterone itself mediates the uterine progestational response via interaction with a specific receptor system.

Considerable evidence has accumulated in support of the receptor hypothesis of steroid hormone action. According to this concept, the binding of steroid hormone by a specific cytoplasmic receptor protein initiates the intracellular translocation of hormone-receptor complex to responsive sites in the target cell (1). For estrogen (estradiol-17β), hormone binding and transport of the estradiol-receptor complex to active sites in the nuclear chromatin takes place without significant metabolism of the steroid molecule (2). In contrast, androgens are actively metabolized within the target cell. After entry into the target cell, testosterone is converted to a potent metabolite, dihydrotestosterone, which then interacts with a specific receptor molecule (3).

The mechanism of progesterone (pregn-4-ene-3,20-dione) action remains to be clarified. Specific progesterone-binding macromolecules have been demonstrated in the cytosol fraction of avian (4–6) and mammalian (7–15) target tissues. However, after progesterone uptake, there is a rapid metabolism of this steroid to a variety of metabolites (16–22). Several possibilities must be considered at this time to account for the mechanism of progestin action. First, there is evidence suggest-

Trivial names: progesterone, pregn-4-ene-3,20-dione; dihydroprogesterone, 5α-pregnane-3,20-dione; pregnanolone, 3α-hydroxy-5α-pregnane-20-one.
Buffers. Buffer A contained 50 mM Tris- HCl (Schwarz/ Mann, ultrapure), 1 mM EDTA, 12 mM triethanol (Sigma), pH 7.5. Buffer B was 10 mM Tris - HCl, 1 mM EDTA, 12 mM triethanol, pH 7.5. Glyceraldehyde (reagent, Matheson, Cole- man & Bell) was added to buffers A and B at a concentration of either 10% (v/v) or 30% (v/v) as indicated.

Uterine Cytosol. Uterine tissue was homogenized in 4 volumes (v/w) of buffer A + 10% glyceraldehyde at 2°C with a Polytron Pt-10 (Brinkmann Instruments), and cytosol was prepared exactly as described before (15). Cellular pellets were analyzed for DNA; calf thymus DNA was the standard (28).

Succrose-Glycerol Gradient Centrifugation. Linear sucrose gradients (5-20% in buffer B + 10% glyceraldehyde) were prepared in 5-ml polycarbonate tubes with a Beckman gradient former. Cytosol, diluted 1/4, (v/w) with buffer A + 10% glyceraldehyde, was incubated for 15 min with unlabeled cortisol (1.1 x 10⁻⁴ M) at 4°C before labeling with [H]-labeled steroid. This was done to saturate binding sites on plasma contaminants such as corticosteroid-binding globulin. Cytosol was then incubated with 2.1 x 10⁻⁴ M of either [1,2-3H]progesterone or [1,2-3H]dihydroprogesterone for 2 hr at 4°C. A 0.2-ml fraction was layered on each gradient. Gradients were centrifuged at 22°C at 202,000 X g for 18 hr in a model L2-65B preparative ultracentrifuge (Beckman). Gradients were fractionated from the bottom (22 fractions/gradient), and the radioactivity content of each fraction was counted as before (15). Sedimentation coefficients were estimated by the method of Martin and Ames (29) with bovine serum albumin as the standard. A 4.2S sedimentation coefficient was assumed for bovine serum albumin.

Scatchard Plot Analysis. Uterine cytosol was diluted 1/16 (w/v) with buffer A + 30% glyceraldehyde. Measurement of the specific progesterone-binding capacity of uterine cytosol fractions was carried out exactly as described previously (15). Specific binding of [H]-progesterone was the difference between total binding and nonspecific binding. Specific binding data were analyzed according to Scatchard (30). The relationship between the bound to free ratio and the concentration bound (nM) was subjected to linear regression analysis (15) providing a correlation coefficient (r), slope (m = Kₐ) and the concentration of progesterone receptor sites (pmol/ml of cytosol). In addition, proestrous uterine cytosol was tested for specific [H]-dihydroprogesterone binding with the same assay procedure but modified as follows: (1) [H]-dihydroprogesterone was substituted for [H]-progesterone, and (2) nonspecific binding was determined by means of a series of tubes containing an excess of unlabeled-4 x 10⁻⁴ M in addition to [H]-labeled dihydroprogesterone. The concentration of the labeled steroid was varied between 0.053 x 10⁻⁴ M and 1.03 x 10⁻⁴ M. Statistical treatment of the data was by Student's t-test. Results were considered significant at P < 0.05.

Metabolism of [H]-Progesterone by Uterine Strips. Uteri from estrogens-primed-ovariectomized hamsters were placed in cold Hank's balanced salt solution, pH 7.4 (BSS). Each uteruus was slit longitudinally into two strips per horn. After blotting and weighing, four uterine strips were used per 25-ml flask each containing 3 ml of Hank's solution. Approximately 6 μCi of either [1,2-3H]progesterone or [7α-3H]progesterone was added to each flask in 10 μl of ethanol. Flasks were placed in a platform shaker at 4°C and incubated with gentle agitation for 90 min. Uptake of radioactivity at 4°C was monitored by taking 10-μl aliquots of medium at 0, 15, 30, 45, 60, and 90 min and measuring the disappearance of radioactivity from the medium. Thus, after incubation at 4°C for 90 min, it was estimated that 30% of the [H]-progesterone had been taken up by the strips. Then the radioactive medium was decanted, the strips were rinsed three times in fresh Hank's solution (4°C), and 3 ml of fresh solution were added per flask. The uterine strips were then incubated in a Dubnoff shaker at 37°C under 95% O₂ and 5% CO₂ with agitation at 60 strokes/min. At various times of incubation, the flask contents were chilled in an ice bath. The medium was collected, and the uterine strips were washed twice with cold Hank's solution. The washes were combined with the incubation medium, and radioactive steroids were extracted three times from the medium with 2 volumes of ethyl acetate each time. Uterine strips were placed in 6 ml of ethanol-acetone (1:1, v/v) for at least 24 hr. Then the strips were pulverized with a ground-glass homogenizer, and the tissue fragments were extracted with ethanol-acetone solution (27).

Thirty micrograms of each of authentic progesterone, 3α-hydroxy-5α-pregn-20-one (pregnanolone), and dihydroprogesterone were added as carrier steroids to each extract. The extracts were taken to dryness, dissolved in 1 ml of methanol, and two 10-μl aliquots were removed for the determination of the initial specific activity (dpm/μg of carrier steroid) (27). Each extract was purified by thin-layer chromatography with narrow plates (5 x 20 cm) coated with silica gel HF-254+366 (Brinkmann) in chloroform-acetone (9:1, v/v) (17, 27). The distribution of radioactivity on the plates was determined with a Nuclear Chicago model 1002 radiochromatogram scanner. Carrier steroid spots were located by the Rₚ values of standards and by direct visualization under UV light. Zones corresponding to the migration of dihydroprogesterone, progesterone, and pregnanolone were eluted individually with ethyl acetate-methanol (1:1, v/v). Each eluate was reduced to dryness, and the residue was acetylated overnight (31). Each steroid fraction was rechromatographed on thin-layer plates in chloroform-ethyl acetate (13:1, v/v) (27). Plates were scanned for the distribution of radioactivity as before, and the zone corresponding to the migration of authentic steroid standard (either dihydroprogestosterone, progesterone, or pregnanolone acetate) was eluted, washed three times with distilled water, and taken to dryness. Each sample was dissolved in 100 μl of ethyl acetate, and two 10-μl aliquots were removed for the measurement of the radioactivity recovered (27). The remainder of the sample was used for determining the recovery of carrier steroid by gas-liquid chromatography; 1% QF1 or 1% OV17 columns were used (27, 32). Radiochemical purity of the samples was established by fraction collection of the column effluent during chromatography as described elsewhere (32). A single radioactive peak was associated with the carrier steroid peak in all cases. The percentage of pregnanolone or dihydroprogesterone produced was calculated from the final specific activity (dpm recovered per μg of carrier steroid recovered) divided by the initial specific activity x 100. The percentage of progesterone utilized equaled 100—(final specific activity + initial specific activity x 100). The amount of progesterone utilized or metabolite produced was calculated from this percentage and from the initial tissue concentration of [H]-progesterone (ng/g of tissue).

RESULTS

Properties of binding components for [H]-progesterone and [H]-dihydroprogesterone in uterine cytosol

A specific progesterone-binding macromolecule with the properties of a hormone receptor was demonstrated previously
in the hamster uterine cytosol fraction (15). This substance exhibited a 6–7S sedimentation coefficient upon density gradient centrifugation in 5–20% sucrose gradients containing 10% glycerol. It possessed a high affinity for progesterone (equilibrium association constant, $K_A = 1 \times 10^8 \text{ M}^{-1}$) as measured by Scatchard plot analysis of specific [H]progesterone-binding data. The receptor showed considerable hormonal specificity in that estrogen, androgens, and corticosteroids failed to compete effectively for progesterone-binding sites. In addition, the receptor showed a greater binding affinity for progesterone than for progesterone metabolites.

In the present study, we measured the specific progesterone-binding capacity of hamster uterine cytosol prepared at the proestrous stage of the estrous cycle (Fig. 1A). In agreement with previous results (15), proestrous cytosol contained a significant quantity of specific progesterone-receptor sites (Table 1). However, specific binding of [H]dihydropro-

**Table 1. Specific progesterone-binding capacity of uterine cytosol fractions**

<table>
<thead>
<tr>
<th>Steroid treatment</th>
<th>Progesterone binding sites</th>
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<tbody>
<tr>
<td></td>
<td>pmol/ml of cytosol</td>
</tr>
<tr>
<td>None (control)</td>
<td>2.83 ± 0.12</td>
</tr>
<tr>
<td>Cortisol</td>
<td>3.16 ± 0.14</td>
</tr>
<tr>
<td>Dihydroprogesterone</td>
<td>2.84 ± 0.15</td>
</tr>
<tr>
<td>Progesterone</td>
<td>1.35 ± 0.08*</td>
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* $P < 0.01$, steroid treatment compared to control. Proestrous hamsters were pretreated for 1 hr with 1 mg of steroid per animal. Each value represents the mean ± SEM for four determinations.

[FIG. 1. Scatchard plots of [H]progesterone- and [H]dihydroprogesterone-binding data in uterine cytosol obtained from proestrous hamsters. (A) [H]Progesterone binding (O—O) demonstrates the presence of a limited quantity of high affinity ($K_A = 1 \times 10^8 \text{ M}^{-1}$) binding sites. [H]Dihydroprogesterone binding (●—●) is nonsaturable and nonspecific. (B) Specific [H]progesterone binding in proestrous uterine cytosol after pretreatment in vivo with unlabeled cortisol (O—O), dihydroprogesterone (△—△), or progesterone (●—●). The number of binding sites relative to tissue weight was obtained from the intercept on the abscissa. Each point represents the mean of duplicate determinations.

Fig. 2. Sucrose-glycerol gradient centrifugation. Distribution of radioactivity after centrifugation (18 hr at 202,000 $\times g$, $2^\circ$) of [H]-labeled uterine cytosol fractions on 5–20% sucrose gradients containing 10% glycerol. The sedimentation peak of bovine serum albumin (arrows) run under identical conditions is indicated above (4.2 S). Proestrous uterine cytosol was used after in vivo pretreatment for 1 hr with unlabeled cortisol (O—O), dihydroprogesterone (△—△), or progesterone (●—●). (A) [H]Progesterone-labeled cytosol; (B) [H]dihydroprogesterone-labeled cytosol.

[Uterine Progesterone Receptor 4343]
stromed the material sedimenting in the 6-7S region of the gradient was progesterone receptor, but the identity of components sedimenting in the 4-5S region has not been established (15). Thus, the present results are consistent with a 6-7S component that is a receptor responsive to progesterone action but not to dihydroprogesterone or cortisol action at the dosage used. The loss of [3H]progesterone binding from the 6-7S region of the sucrose-glycerol gradient after in vivo progesterone administration suggested that receptor material had been removed from the cytosol fraction. This interpretation is supported by the finding of a significant reduction in the concentration of progesterone receptor sites after progesterone treatment (Table 1).

Sucrose-glycerol gradient centrifugation of [3H]dihydroprogesterone-labeled uterine cytosol revealed a major binding peak with an approximate sedimentation coefficient of 4 S (fractions 7 + 8, Fig. 2B). This region of the gradient is occupied by steroid-binding proteins derived from plasma such as corticosteroid-binding globulin and albumin as well as other unidentified steroid-binding components (15). Therefore, the tissue specificity of binding material in this region can be questioned, and the steroid specificity of 4-5S components is uncertain. Inasmuch as none of the steroid pretreatments affected the amount of [3H]dihydroprogesterone binding in the 4S region of the gradient, we conclude that the 4S peak of this binding represents nonspecific binding material(s). Thus, these results coupled with those from the Scatchard plot analysis demonstrate that hamster uterine cytosol does not contain a specific receptor system for dihydroprogesterone.

**[3H]Progesterone metabolism by uterine strips in vitro**

Uterine strips containing two different concentrations of [3H]-progesterone were incubated for different times at 37°C (Fig. 3). From the results, it appeared that enough [3H]progesterone was present to saturate the receptor system in both cases. However, the amount of nonspecific [3H]progesterone binding was estimated to be greater in the first experiment (236 ng/g of tissue) than in the second (40 ng/g of tissue).

There was a rapid metabolism of [3H]progesterone during the first 30 min of incubation, and this was followed by a slower [3H]progesterone utilization from 30 to 240 min (Fig. 3). Progesterone metabolism (ng/g of tissue) was greater in those strips with the higher initial concentration of [3H]-progesterone (Fig. 3A) than in those containing the lower concentration (Fig. 3B). Two radioactive metabolites of [3H]-progesterone were identified on the basis of (I) chromatographic mobility in two different thin-layer chromatography systems, and (2) a retention time identical to that of authentic steroid standard determined by gas-liquid chromatography with 1% QF1 and 1% OV17 columns. The major metabolite formed during incubation was 3α-hydroxy-5α-pregn-20-one (pregnanolone), and smaller amounts of 5α-pregnane-3,20-dione were produced (Fig. 3). In addition, a small quantity of polar metabolite(s) was observed to accumulate in the medium, but this material was not identified.

The time-course of pregnanolone production followed the pattern observed for progesterone utilization throughout the incubation period in both experiments (Fig. 3). In contrast, dihydroprogesterone production increased during the first 30 min and then a plateau was observed for the remainder of the incubation period. The maximal amount (ng/g of tissue) of dihydroprogesterone which accumulated was related to the initial tissue concentration of [3H]progesterone. Since the progesterone receptor titer was the same in both experiments, it seems pertinent that more dihydroprogesterone was produced in tissue containing the higher level of nonspecifically bound [3H]progesterone (compare dihydroprogesterone production in Fig. 3A and B).

**DISCUSSION**

Information derived from studies of the action of aldosterone, estrogen, and androgen have shown that in all cases the target tissues contain a limited quantity of soluble receptor protein which selectively binds hormone with high affinity (1). The present results confirm the existence of a specific progesterone receptor in the hamster uterine cytosol fraction (15). Progesterone receptor sites were depleted from the cytosol fraction in response to in vivo progesterone action, but no response was elicited by treatment with an equivalent amount of dihydroprogesterone or cortisol. The competitive binding activity of dihydroprogesterone and cortisol for progesterone
receptor sites is 20% and <1%, respectively, as compared to the activity of progesterone (15). From this, it would appear that the receptor has a considerably greater binding affinity for progesterone than for dihydroprogesterone. Thus, the present findings are consistent with a relationship between the binding affinity of the progesterone receptor for a steroid and the biological activity of the steroid. Such a relationship has been observed in the case of estrogen and androgen receptor systems (33, 84). Furthermore, there is evidence indicating that the translocation of hormone-receptor complex from the cytosol fraction to active intranuclear sites is a primary event in the mechanism of hormone action (2). The progesterone-induced depletion of receptor sites from the uterine cytosol fraction may represent an early step in the mechanism of progestin action, i.e., transport of progesterone-receptor complex to acceptor sites in a particulate fraction.

Although dihydroprogesterone has been suggested as a possible intracellular mediator of progestin action (19, 25), we were unable to demonstrate a specific binding macromolecule for this steroid in the hamster uterine cytosol fraction. The apparent lack of a specific receptor might imply that the mechanism of progestin action in the uterus does not involve conversion of progesterone to an active metabolite as appears to be so for androgen target tissues (26). However, hamster uterine tissue metabolized progesterone to both dihydroprogesterone and pregnanolone during incubation of uterine strips which had been preloaded with [3H]progesterone. Pregnanolone was the major metabolite produced by hamster uterus, and this product accumulated during incubation in proportion to the amount of progesterone utilized. In contrast, the amount of dihydroprogesterone which accumulated during incubation increased only during the first 30 min. Also, the production and accumulation of this metabolite appeared to be related to the amount of nonspecifically-bound progesterone which was available for metabolism rather than to the amount which was specifically bound.

In the rat, dihydroprogesterone and pregnanolone were identified as the principal progestrone metabolites formed in the uterus (8, 16, 19). When progestational activity of these two metabolites was tested in the rat, dihydroprogesterone and pregnanolone possessed little biological activity in the uterus (35). The uterotrophic activity of dihydroprogesterone has been tested in the hamster, and a similar lack of effect was observed (36). Our failure to detect a specific receptor system for this metabolite in the hamster uterus coupled with bioassay results indicating that it is an inactive progesterone metabolite lead us to the conclusion that progesterone is the primary regulator of the progestational response in the uterus. The present results with the hamster are in agreement with a previous study with the rat (20) which indicated that uterine progesterone metabolism probably represents a mechanism for clearing active hormone (progesterone) from the target cell. This conclusion does not exclude the possibility that progesterone metabolites may play an active role in the mechanism of hormone action in nonuterine target tissues. Dihydroprogesterone stimulates lutetizing hormone release in both the rat (37) and hamster (38), and it is capable of stimulating avidin synthesis in the estrogen-primed chick oviduct (22). Therefore, dihydroprogesterone may be involved in the mechanism of progestin action in neural and oviduct tissue, but the present results indicate that this is not so in the uterus.

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